Identification of Free Radical Formation and F_2-Isoprostanes in Vivo by Acute Cr(VI) Poisoning

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We previously reported the detection of a carbon-centered radical adduct of α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone (POBN) in the bile of rats acutely poisoned with Cr(VI) utilizing an electron spin resonance spin-trapping technique. These former studies suggested that the free radical metabolite was derived from a polyunsaturated fatty acid. The present studies were undertaken to further characterize this radical adduct and to determine whether its formation is associated with enhanced lipid peroxidation in vivo. This report demonstrates that electron spin resonance (ESR) spectra with hyperfine coupling constants a^N of 15.71 G and a_β^H of 2.90 G were present in bile from Cr(VI)-poisoned rats. We found out that virtually identical ESR spectra were obtained when authentic POBN−pentyl radical adducts generated from the reaction of POBN with either pentylhydrazine or linoleic or arachidonic acid with lipoxygenase were added to bile. The hyperfine coupling constants for the POBN−pentyl radical adducts added to bile were as follows: $a^N = 15.85$ G and $a_\beta^H = 2.60$ G for the reaction between pentylhydrazine and POBN; $a^N = 15.72$ G and $a_\beta^H = 2.61$ G for the reaction between arachidonic acid, lipoxygenase, and POBN; and $a^N = 15.85$ G and $a_\beta^H = 2.85$ G for the reaction between linoleic acid, lipoxygenase, and POBN. In addition, the formation of this radical adduct was associated with lipid peroxidation as quantified by increases in F_2-isoprostane levels in bile. These studies, therefore, provide additional evidence that acute Cr(VI) poisoning is associated with enhanced generation of F_2-isoprostanes in vivo and tentatively identify the radical species that is produced as the POBN−pentyl radical adduct.

Introduction

Studies have shown that chromates can have serious genotoxic and carcinogenic effects in humans and animals (*1*−*3*). According to the uptake−reduction model, Cr(VI) compounds (e.g., chromates and dichromates) readily cross cell membranes and are reduced intracellularly (*4*). A number of biologically relevant compounds have been shown to reduce Cr(VI), including thiols, ascorbate, vitamin B_2, cytochrome P450, and complexes within the mitochondrial electron transport system (*5*). Generally, under physiological conditions, Cr(VI) is capable of reacting with redox enzymes and biochemical reductants to produce Cr(V), Cr(IV), and thiyl and hydroxyl radicals (*6*). The valence of chromium is important with regard to its carcinogenic and mutagenic properties. For example, relative to Cr(VI), Cr(III) exhibits benign chemical and biological properties which can be explained by the difference in their abilities to cross the cell membrane and by the difference in their reactivities with cellular components (*5*).

Carcinogenic transition metals, including Cr(VI), have been shown to induce lipid peroxidation (*7*−*11*). Since lipid peroxidation is thought to result in membrane damage and to play an important role in tissue injuries induced by a number of xenobiotics, it is possible that certain manifestations of Cr(VI) toxicity and carcinogenicity are due to enhanced lipid peroxidation (*12*−*14*). In studies of the effects of Cr on inducing lipid peroxidation, Yonaha and co-workers (*14*) have demonstrated the inhibitory action of trivalent and hexavalent Cr at low concentrations on lipid peroxidation in rat liver microsomes and a prooxidant effect of hexavalent chromium at higher concentrations. It has also been reported that the extent of lipid peroxidation in mouse liver was significantly increased 24 and 48 h after Cr(VI) exposure (*12*). Another report (*13*) demonstrated that lipid peroxidation induced by Cr(VI) was enhanced significantly in isolated hepatocytes pretreated with glutathione-depleting agent. These data agree with those obtained by Coudray and co-workers (*15*), who used rat heart homogenate to show that Cr(VI), but not Cr(III), is able to enhance lipid peroxidation.

The studies cited above, in which the prooxidant effects of Cr(VI) were examined, quantified lipid peroxidation by assessing thiobarbituric acid-reacting substances (TBARS[1]). While this method is simple to perform, it is quite unreliable, particularly when applied to animal or human biological fluids or tissues (*16*). On the other hand, we previously discovered a series of prostaglandin

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[1] Abbreviations: ESR, electron spin resonance; 4-POBN, α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone; LC/ESI/MS, liquid chromatography/electrospray ionization mass spectrometry; GC/NICI-MS, gas chromatography/negative ion chemical ionization mass spectrometry; TBARS, thiobarbituric acid-reacting substances.

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(PG) F_2-like compounds, termed F_2-isoprostanes, derived in vivo from the free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme. We have performed a large number of studies (*17*, *18*) which suggest that the quantification of these compounds represents an accurate measure of lipid peroxidation both in vivo and in vitro.

Previously, we reported the detection of a lipid-derived free radical adduct of POBN in rats acutely poisoned with Cr(VI) (*19*). In an effort to further characterize this radical species, we performed a series of spin-trapping experiments and report herein the tentative identification of this radical as a lipid-derived pentyl radical, which can be detected in bile collected from chromium-poisoned rats. We also sought to determine whether formation of this radical was associated with enhanced lipid peroxidation by quantifying F_2-isoprostanes and measuring TBARS in the same bile samples. We report that the administration of Cr(VI) was associated with marked increases in the extent of excretion of F_2-isoprostanes in bile, supporting the hypothesis that generation of free radicals by Cr(VI) is associated with enhanced lipid peroxidation in vivo.

Materials and Methods

α-(4-Pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) and soybean lipoxygenase type I (containing 140 000 units/mg) were purchased from Sigma (St. Louis, MO). Potassium dichromate [Cr(VI)] was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Arachidonic acid and linoleic acid (>99%) were purchased from Nu-chek Prep. (Elysian, MN). Ethylhydrazine oxalate was purchased from Fluka Chemical Co. (Ronkonkoma, NY). All chemicals used were commercial products of the highest grade available.

In Vivo Studies. Nonfasted male Sprague-Dawley rats (450 ± 75 g) were anesthetized with Nembutal (0.1 mL/100 g ip), the level of which was maintained throughout the experiments. Bile ducts were cannulated before treatment using 10 cm segments of PE 10 tubing. The rats were given an intragastric injection of potassium dichromate (5 mmol/kg). Control rats received an intragastric injection of saline.

In all in vivo experiments, 40 min bile samples from 0 to 40, 40 to 80, and 80 to 120 min after Cr(VI) administration were collected in plastic Eppendorf tubes. Samples were frozen immediately on dry ice and stored at −70 °C until analysis.

In Vitro Studies. The pentyl radical adduct was generated via the decomposition of pentylhydrazine. The reaction mixtures contained 50 mg of 4-POBN, 20 mg of pentylhydrazine oxalate, and 0.2 mM $CuCl_2$ in 5 mL of 0.05 M carbonate buffer (pH 10.0). After nitrogen gas was bubbled through the carbonate buffer, pentylhydrazine oxalate and $CuCl_2$ were added. The reactions were performed anaerobically for 2 h at 25 °C. Identification of the 4-POBN−pentyl radical adduct was carried out using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI/MS), as described previously (*20*).

Since the identification of the same radical adducts was reported recently for the reactions of unsaturated fatty acids with soybean lipoxygenase (*21*), we applied these reactions to the investigation of the 4-POBN−pentyl radical adducts in bile. Reaction mixtures contained 5 mL of 0.05 M borate buffer (pH 9.0), 0.5 mL of 1 M 4-POBN, 0.025 mL of a 25 mg/mL solution of linoleic or arachidonic acid dissolved in ethanol, and 0.015 mL of soybean lipoxygenase (1.4×10^8 units/mL). The reactions were allowed to proceed at 25 °C in the absence of light for 4 h. To obtain 4-POBN radical adducts in the appropriate solvent environment, 0.1 mL of the reaction mixture was added to 0.4 mL of bile from untreated control rats.

ESR Measurement. ESR spectra were recorded at room temperature using a quartz flat cell and a Varian E−109 ESR spectrometer fitted with a TM_{110} cavity operating at 9.33 GHz, a 20 mW microwave power, and a 100 kHz modulation frequency. Spectra were recorded on an IBM-compatible computer interfaced with the spectrometer. The ESR spectral simulations were performed using an automatic optimization procedure (*22*).

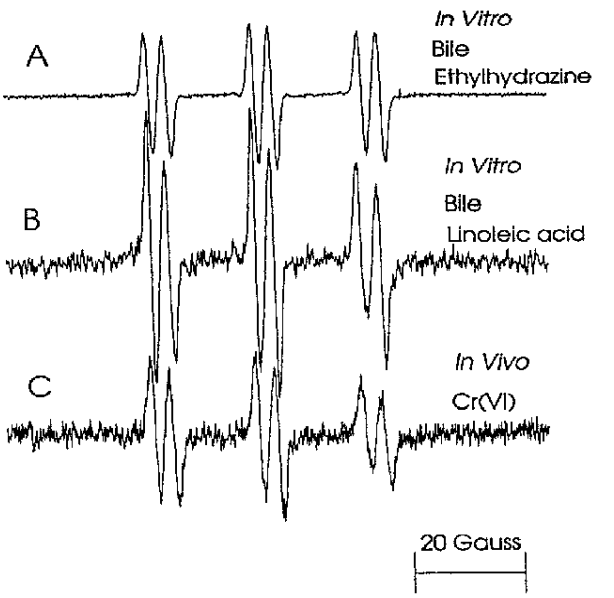

Figure 1. ESR spectra of radical adducts detected in bile from untreated and Cr(VI)-treated rats. (A) ESR spectrum of a POBN radical adduct formed through the decomposition of pentylhydrazine oxalate. The reaction mixture contained 50 mg of POBN, 20 mg of pentylhydrazine oxalate, and 0.2 mM $CuCl_2$ in 5 mL of 0.05 M carbonate buffer (pH 10.0). After the reaction was performed for 2 h at 25 °C under nitrogen, 0.1 mL was added to 0.4 mL of bile from untreated rats. Instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 0.128 s; and scan time, 4 min. (B) ESR spectrum of POBN radical adducts formed in the reaction mixture of 2 mL of borate buffer (0.2 M, pH 9.0), 0.1 M POBN, 760 μM 13-(hydroperoxy)linoleic acid, and 13 600 units/mL soybean lipoxygenase. After the reaction was performed at 25 °C under nitrogen in the dark, 0.1 mL was added to 0.4 mL of bile from untreated rats. Instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 0.5 s; and scan time, 8 min. (C) ESR spectrum of radical adducts detected in bile from rats 120 min after administration of potassium dichromate (3 mmol/kg, ig) and POBN (8 mmol/kg, ip). Instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 1 s; and scan time, 16 min.

Quantification of F_2-Isoprostanes in Bile Samples. Analyses of F_2-isoprostanes by gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) were performed with 800 μL bile samples as described previously utilizing stable isotope dilution methods (*23*).

Results

When an intragastric injection of potassium dichromate (3 mmol/kg) was administered to rats followed by an intraperitoneal injection of 4-POBN, a strong six-line ESR signal was detected in the bile (Figure 1C) (*19*). The assignment of this radical adduct(s) as a carbon-centered, endogenous, lipid-derived adduct was strengthened through further in vitro experiments.

The reaction mixtures containing authentic pentyl radical adduct of 4-POBN were added to bile from untreated rats and analyzed using ESR spectroscopy. For the complete reaction mixture, typical six-line ESR signals were observed (Figure 1A). The hyperfine cou-

Table 1. Hyperfine Coupling Constants of Radical Adducts Obtained from Bile[a]

radical adducts	hyperfine coupling constants (G) a^N	a_β^H	source
POBN/$^{\bullet}CH_2(CH_2)_3CH_3$ (from pentylhydrazine)	15.85 ± 0.03	2.60 ± 0.04	this work[b]
POBN/$^{\bullet}$L (from lipoxygenase with linoleic acid)	15.76 ± 0.04	2.85 ± 0.09	this work[b]
POBN/$^{\bullet}$L (from lipoxygenase with arachidonic acid)	15.72 ± 0.01	2.61 ± 0.09	this work[b]
POBN/$^{\bullet}CH_2(CH_2)_3CH_3$ (from lipoxygenase with linoleic acid)	15.80	2.60	*20*[c]
POBN/$^{\bullet}$L (from LOOH)	15.64	2.73	*24*[b]
POBN/$^{\bullet}$L [from Cr(VI)]	15.71 ± 0.04	2.90 ± 0.13	*19*[b]

[a] Values ± SE were from three different experiments. [b] In bile, the experimental conditions were as follows. For radical adducts from pentylhydrazine, the reaction mixture contained 50 mg of POBN, 20 mg of pentylhydrazine oxalate, and 0.2 mM $CuCl_2$ in 5 mL of 0.05 M carbonate buffer (pH 10.0). After the reaction was performed for 2 h at 25 °C under nitrogen, 0.1 mL was added to 0.4 mL of bile from untreated rats. For radical adducts from lipoxygenase with lineolic (arachidonic) acid, the reaction mixture contained 2 mL of borate buffer (0.2 M, pH 9.0), 0.1 M POBN, 760 μM 13-(hydroperoxy)linoleic (arachidonic) acid, and 13 600 units/mL soybean lipoxygenase. After the reaction was performed at 25 °C under nitrogen in the dark, 0.1 mL was added to 0.4 mL of bile from untreated rats. [c] In buffer, the same experimental conditions were used but without bile.

pling constants for the POBN−pentyl radical adducts added to bile were as follows: a^N = 15.85 G and a_β^H = 2.60 G for the reaction between pentylhydrazine and POBN (Figure 1A); a^N = 15.72 G and a_β^H = 2.61 G for the reaction between arachidonic acid, lipoxygenase, and POBN; and a^N = 15.85 G and a_β^H = 2.85 G for the reaction between linoleic acid, lipoxygenase, and POBN. Since these hyperfine coupling constants were significantly close to, if not the same as, those identified earlier (*20*), we identified this POBN adduct detected in bile as POBN−pentyl radical adduct.

Further evidence for the generation of POBN−pentyl radical adduct has also been provided when 24 h reaction mixtures of linoleic or arachidonic acid with soybean lipoxygenase were analyzed using HPLC/ESR spectroscopy (*20*, *21*). Iwahashi and co-workers (*20*) have found that authentic POBN−pentyl radical adduct obtained from the reaction between pentylhydrazine and POBN gave the same mass spectrum as the major product obtained from the reaction of linoleic acid or arachidonic acid with lipoxygenase in the presence of POBN. To investigate the same radical adducts generated in bile, additional experiments were carried out in which incubation mixtures of linoleic (or arachidonic) acid, lipoxygenase, and POBN were added to bile from control rats. Figure 1B shows the spectrum detected from bile containing the reaction mixture of linoleic acid with soybean lipoxygenase. Since there were no statistically significant differences in the signal intensity of the ESR spectrum from bile recorded at 40, 80, and 120 min after Cr(IV) poisoning, Figure 1C shows the spectrum detected in the bile of a rat 120 min after treatment with Cr(VI) (*19*). When arachidonic acid reacted with lipoxygenase, we detected an ESR signal from bile like those shown in parts A and B of Figure 1 (data not shown). The hyperfine coupling constants for the 4-POBN radical adducts detected in the bile are listed in Table 1. As

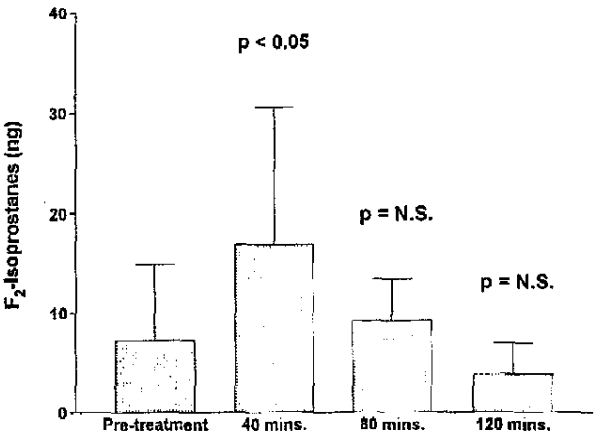

Figure 2. Levels of F_2-isoprostanes from animals before (n = 10) and 40 (n = 13), 80 (n = 12), and 120 min (n = 9) after administration of potassium dichromate (3 mmol/kg, ig). Values are means ± SD. Levels are expressed as nanograms of F_2-isoprostanes per 40 min bile sample. Data were analyzed for statistical significance using analysis of variance (ANOVA), and comparisons were made between the level of F_2-isoprostanes at each time interval after potassium dichromate administration and before treatment. Differences were considered significant if $P < 0.05$. n is the number of animals used in each group.

shown in Table 1, a^N and a_β^H values from our system are in agreement with the assignment of POBN−pentyl radical adduct formed by the reductive decomposition of fatty acid hydroperoxides (*20*, *21*, *24*).

The hyperfine coupling constants for the radical adducts detected previously in the bile during Cr(VI) poisoning (*19*) are virtually identical to those of POBN−pentyl radical adducts detected in this study. Therefore, on the basis of the hyperfine coupling constants, the radical adduct detected in bile from Cr(VI) poisoning can be tentatively identified as POBN−pentyl radical adduct, most probably from the decomposition of lineolic and archidonic acid hydroperoxides.

To determine whether the radical generated by Cr(VI) poisoning in rats is associated with increased lipid peroxidation in vivo, F_2-isoprostane levels were quantified in bile obtained from rats 0−40, 40−80, and 80−120 min after Cr(VI) administration. Levels of F_2-isoprostanes were markedly increased over baseline at 0−40 min ($p < 0.05$) and returned to normal thereafter, suggesting that administration of Cr(VI) rapidly induces lipid peroxidation in vivo after administration (Figure 2). However, Cr(VI) poisoning did not have an effect on TBARS measured spectrophotometrically in bile samples and liver microsomes (data not shown).

Discussion

We have previously proposed that the free radical detected after Cr(VI) administration to rats is a carbon-centered radical likely derived from a polyunsaturated fatty acid. In this study, we have further characterized this radical as a pentyl radical.

Reports in the literature concerning the ability of Cr(VI) to induce lipid peroxidation appear to be contradictory in relation to its induction or inhibition (*12*, *13*, *15*). The volatile hydrocarbon pentane has been detected during the peroxidation of PUFAs (*25*−*28*). According to Garssen and co-workers (*28*), β-scission of alkoxyl radical (LO$^{\bullet}$) occurs because of one-electron reduction

from the hydroperoxide (LOOH) and results in the formation of an aldehyde and pentyl radical. The pentyl radical is proposed to form pentane by abstracting hydrogen from one of the surrounding molecules. Thus, detecting the pentyl radical as a precursor of pentane formation is essential proof of the lipid peroxidation process.

Evidence for the role of lipid peroxidation in the toxicity of Cr is provided by our ESR data obtained when an authentic pentyl radical adduct was produced in the bile. This assignment is supported by these findings, since the hyperfine coupling constants of the POBN–pentyl radical adduct generated in vitro and added to bile are almost identical to those of the radical adduct detected in bile following Cr(VI) poisoning. Previously reported data for POBN–pentyl radical adduct hyperfine couplings were determined in buffer (Table 1). Thus, our present data support the participation of fatty acid-derived free radicals in the toxic effect of potassium dichromate. In addition, formation of this radical is associated with enhanced lipid peroxidation as assessed by an increased level of F_2-isoprostane formation in the bile in vivo. Since no changes were found in TBARS, we attribute these results to the suggestion that isoprostane measurement appears to be a more reliable indication of lipid peroxidation in vivo than TBARS. The data indicate that Cr(VI) induces isoprostane generation, thereby providing strong evidence of lipid peroxidation by Cr in the liver. It has been reported that quantification of F_2-isoprostanes represents a reliable and useful approach for assessing lipid peroxidation in vivo (*29*). These lipid peroxidation products are primarily formed in situ on phospholipids because of free radical-catalyzed peroxidation of arachidonic acid and are subsequently released in free form by phospholipases (*29*). Although the extent of formation of isoprostanes has been shown to increase dramatically in animal models of free radical injury (*30*), reports in the literature show that factors which modulate the generation of F_2-isoprostanes have not been completely explored. In this study, we have shown the enhanced formation of F_2-isoprostanes in bile by acute Cr(VI) poisoning, supporting the hypothesis that Cr(VI) poisoning induces an oxidant stress in vivo. Evaluation of in vivo chromate induction of lipid peroxidation by the enhanced generation of isoprostanes and lipid-derived radicals should allow an assessment of the significance of lipid peroxidation in experimental Cr metabolism, toxicosis, and carcinogenesis. In addition, our study provides a better understanding of the reaction processes involved in lipid peroxidation, which can be important in predicting and preventing potential chromate toxicity and carcinogenicity.

In summary, these studies further identify the radical metabolites produced in vivo by acute Cr poisoning as pentyl radical derived from PUFA and provide definitive evidence that Cr(VI) poisoning induces lipid peroxidation in vivo.

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